Journal of Experimental Botany, Vol. 58, No. 15/16, pp. 4245–4255, 2007
doi:10.1093/jxb/erm284
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance

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Received 10 August 2007; Revised 21 October 2007; Accepted 23 October 2007

Abstract

The accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress conditions, including both salinity and oxidative stress. In this work, a possible causal link between the ability of contrasting barley genotypes to accumulate/synthesize compatible solutes and their salinity stress tolerance was investigated. The impact of H_2O_2 (one of the components of salt stress) on K^+ flux (a measure of stress 'severity') and the mitigating effects of glycine betaine and proline on NaCl-induced K^+ efflux were found to be significantly higher in salt-sensitive barley genotypes. At the same time, a 2-fold higher accumulation of leaf and root proline and leaf glycine betaine was found in salt-sensitive cultivars. The total amino acid content was also less affected by salinity in salt-tolerant cultivars. In these, potassium was found to be the main contributor to cytoplasmic osmolality, while in salt-sensitive genotypes, glycine betaine and proline contributed substantially to cell osmolality, compensating for reduced cytosolic K^+. Significant negative correlations (r= –0.89 and –0.94) were observed between Na^+-induced K^+ efflux (an indicator of salt tolerance) and leaf glycine betaine and proline. These results indicate that hyperaccumulation of known major compatible solutes in barley does not appear to play a major role in salt-tolerance, but rather, may be a symptom of salt-susceptibility.

Key words: Glycine betaine, *Hordeum vulgare* L., potassium flux, proline, reactive oxygen species, salinity.

Introduction

Salinity is one of the major abiotic factors limiting global agricultural productivity, rendering an estimated one-third of the world's irrigated land unsuitable for crops (Frommer *et al*., 1999). Salt stress in plant cells is primarily caused by a combination of osmotic and ionic stress resulting from high Na^+ concentration in the soil (Hasegawa *et al*., 2000). Metabolic acclimation via the accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress (Hanson and Hitz, 1982; Bohnert and Jensen, 1996; Sakamoto and Murata, 2000; Shabala and Cuin, 2006). Many plant species accumulate significant amounts of glycine betaine, proline, and polyols in response to high salinity (Rhodes and Hanson, 1993; Bohnert *et al*., 1995; Di Martino *et al*., 2003). Multiple functions for these compounds have been suggested. In addition to the conventional role of these compatible solutes in cell osmotic adjustment (Yancey *et al*., 1982; Bray, 1993), they are also suggested to act as low-molecular-weight chaperones, stabilizing the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging ROS (Robinson and Jones, 1986; Smirnoff and Cumbes,

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Abbreviation: ROS, reactive oxygen species.

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1989; McCue and Hanson, 1990; Santoro *et al*., 1992; Bohnert *et al*., 1995; Papageorgiou and Murata, 1995; Shen *et al*., 1997; Hare *et al*., 1998; Mansour, 1998; Noiraud *et al*., 2001). Recently, it was also shown that some of these compatible solutes are very efficient in reducing the extent of K^+ loss in response to both salinity (Cuin and Shabala, 2005, 2007*a*) and oxidative stress (Cuin and Shabala, 2007*b*) in barley and *Arabidopsis* roots.

Different varieties of a particular plant species exhibit a high degree of variation in salt tolerance (Epstein *et al*., 1980; Chen *et al*., 2007) and there are various reports on the differential accumulation of glycine betaine and proline among genotypes of cereals (Wyn Jones and Storey, 1978; Rhodes *et al*., 1989; Colmer *et al*., 1995; Yang *et al*., 2003), indicating a possible causal link between these processes. Indeed, the introduction of genes involved in the synthesis of proline, betaines, and polyols into plants contributes to abiotic stress tolerance (Rathinasabapathi, 2000; Chen and Murata, 2002) and numerous genetic engineering attempts have been made to manipulate the biosynthesis pathway of compatible solutes in order to enhance salt tolerance (Rathinasabapathi, 2000; Sakamoto and Murata, 2000; Chen and Murata, 2002).

However, the levels of compatible solutes accumulated in transgenic plants are not high enough to be osmotically significant (Hare *et al*., 1998; Bohnert and Shen, 1999; Sakamoto and Murata, 2000). Thus, exogenous application of compatible solutes has been suggested as an alternative approach to improve crop productivity under saline conditions (Mäkelä *et al*., 1999; Chen and Murata, 2002). External application of low exogenous concentrations of glycine betaine and proline maintained higher K^+ concentration in salt-stressed tomato leaves (Heuer, 2003) and decreased salt-induced K^+ efflux from barley roots (Cuin and Shabala, 2005, 2007*a*). Although some researchers have reported positive correlations between the capacity for glycine betaine and/or proline accumulation and salinity tolerance (Binzel *et al*., 1987; Hare and Cress, 1997; Almansouri *et al*., 1999; Meloni *et al*., 2001), others have challenged the value of these solutes as positive indicators for resistance to salt stress (Delauney and Verma, 1993; Heuer, 2003). Thus, controversies exist as to whether hyperaccumulation of glycine betaine and proline is essential for improving salinity tolerance, or whether it is just a symptom of salt stress. In addition, it cannot be excluded that both mechanisms may coexist, providing some effective ROS scavenging in sensitive cultivars or species, while indicating a symptom of salt stress in tolerant ones. These issues are explored in more detail in this study.

One of the hallmarks of salt stress is a massive K^+ efflux from plant roots (Shabala *et al*., 2003, 2005), affecting cytosolic K^+ homeostasis (Cuin *et al*., 2003; Shabala *et al*., 2006), and therefore growth and survival of the plant. In our previous studies, a strong correlation has been observed between NaCl-induced K^+ efflux and barley salt tolerance, based on variety of physiological and agronomical indices (Chen *et al*., 2005, 2007). This led to the proposition of using K^+ retention as an indicator for barley salt tolerance. Given our previous findings that applied compatible solutes are generally efficient in reducing the extent of K^+ loss in response to both salinity (Cuin and Shabala, 2005, 2007*a*) and oxidative stress (Cuin and Shabala, 2007*b*), and the fact that ROS production is an established component of salt stress signalling (Hasegawa *et al*., 2000; Zhu, 2001; Demidchik and Maathuis, 2007), the possible causal link between the ability of barley to accumulate/synthesize compatible solutes and salinity stress tolerance warrant a thorough investigation. This was the main aim of this study.

Materials and methods

Plant materials and growth conditions

Four barley cultivars: salt-tolerant Numar and ZUG293, and salt-sensitive Gairdner and ZUG403, from the Australian Winter Cereal Collection and Barley Genotypic Collection of Zhejiang University, were used in this study. For K^+ flux experiments, seedlings were grown for 3 d in an aerated hydroponic solution (0.5 mM KCl and 0.1 mM $CaCl_2$) in a dark growth cabinet at 24±1 °C. Seedlings with a root length 70±10 mm were used for measurements. For the greenhouse trial, barley plants were grown in a semi-hydroponic culture as described by Chen *et al*. (2005). The average greenhouse temperature and humidity over the growth season was 23 °C and 57%, respectively. A randomized complete block design was used, with ten replicates for each treatment. Eight seeds were sown and thinned to four healthy seedlings in each pot. Half-strength Hoagland's solution was used in both control and salt-treated plants. Salt treatment was applied at 320 mM NaCl, added gradually with a daily increment of 40 mM NaCl, commencing 3 weeks after sowing. Plants were watered twice daily by an automatic irrigation system through drippers, with about 60 ml of control or saline solution applied each time per pot. After 4 weeks of salt treatment, flag leaf and root samples were collected for HPLC and osmolality measurements, after recording plant height. All other plants were harvested, fresh mass weighed and dry mass determined after 72 h at 65 °C in a Unitherm Dryer (Birmingham, UK). All chemicals were from Sigma-Aldrich (Castle Hill, NSW Australia) unless otherwise specified.

Leaf sap osmolality

One day prior to harvest, four flag leaves of each genotype/treatment were sampled and stored at –20 °C. Flag leaf blade sap was extracted using the freeze–thaw method (Tomos *et al*., 1984) and its osmolality was determined using a vapour pressure osmometer (Vapro, Wescor Inc. Logan, Utah, USA).

Leaf Na^+ and K^+ contents

Dry barley leaves were ground and passed through a 2 mm mesh sieve. Samples were digested in 10 ml 98% H_2SO_4 and 3 ml 30% H_2O_2 for 5 h, essentially as described by Skoog *et al*. (2000). The Na^+ and K^+ contents were determined using a flame photometer.

K^+ flux measurements

Net K^+ fluxes were measured at the root mature zone, about 10 mm from the root tip, using the non-invasive ion-selective microelectrode MIFE™ technique (University of Tasmania, Hobart, Australia),

essentially as described by Shabala *et al*. (1997, 2003). In brief, glass microelectrodes filled with ion-selective cocktail (K^+ 60031, Fluka, Buchs, Switzerland) were moved in slow (10 s cycle, 40 μm amplitude) square-wave by a computer-driven micromanipulator (Patchman NP2, Eppendorf, Hamburg, Germany). Net K^+ fluxes were calculated as described by Newman (2001). In salinity experiments, a 3-d-old seedling was taken from the growth cabinet 1 h prior to measurement and placed in a Perspex measuring chamber with 10 ml solution (80 mM NaCl, 0.5 mM KCl, and 0.1 mM $CaCl_2$). K^+ flux was then recorded after 1 h salt treatment, then an appropriate amount of either proline or glycine betaine was added, and K^+ flux was recorded for a further 15 min. For the H_2O_2 treatments, K^+ flux was measured in the standard bath solution (0.5 mM KCl and 0.1 mM $CaCl_2$) for 10 min followed by another 30 min after the addition of either 1 or 10 mM H_2O_2.

Membrane potential measurements

Conventional KCl-filled Ag/AgCl microelectrodes (Shabala and Lew, 2002; Cuin and Shabala, 2005) with a tip diameter 0.5 μm were used with the MIFE electrometer to measure membrane potential (E_m) from epidermal cells in the root mature zone. Measurements were taken in the standard bath solution from either non-treated roots (controls), or 5–15 min after root exposure to 10 mM H_2O_2. Following cell penetration, E_m was recorded for about 2 min for each measurement. At least four individual plants for either control or H_2O_2-treated roots were measured, with between two and four cell measurements for each individual root.

Determination of compatible solutes

HPLC instrumentation: The high performance liquid chromatography (HPLC) system consisting of a 717Plus autosampler, 600E pump, 996 photodiode array (PDA) detector and Millennium Chromatography Manager software (version 32) (Waters Australia Pty Ltd. Rydalmere, NSW, Australia) was used to quantify levels of compatible solutes in plants. The absorption spectrum of eluted compounds was scanned every second from 190 nm to 400 nm at intervals of 1.2 nm. Microsorb-MV Amino column (250 mm×4.6 mm) and 4.6 mm MetaGuard column were employed (Varian Inc, USA) with the stationary phases at microsorb-MV 100 NH_2 and Polaris NH_2 with particle sizes of 5 μm. The mobile phase with acetonitrile:water in the ratio of 84:16 was filtered through 0.45 μm nylon filter under vacuum with a flow rate at 1.50 ml min^{-1}. The columns were maintained at 30 °C during chromatography.

Sample extraction and purification: Leaf and root samples were freeze-dried and stored below –15 °C until analysis. Samples were extracted as described by Naidu (1998). Leaf and root samples were weighed and placed into 10 ml centrifuge tubes. To each tube, 5 ml of methanol:chloroform:water (60:25:15 by vol.) was added. Tubes were then sealed and heated at 60 °C in a water bath for 2 h. Tubes were then removed and 5 ml of deionized water added. The samples were shaken vigorously for 1 min before centrifugation for 10 min at 4000 rpm. The clear upper layer was purified through strong anion exchange resin beads, then filtered through a 0.22 μm Millex-GS syringe driven filter unit prior to injection into the HPLC.

Glycine betaine, sugars, and polyols: Glycine betaine, sugars, and polyols were determined as described by Naidu (1998). A mixture of standards: glycine betaine, sucrose, glucose, fructose, mannitol, pinitol, and sorbitol, was prepared in methanol:water (50:50, v:v) at 0.5 μg μl^{-1} for glycine betaine and 2.5 μg μl^{-1} for the remaining solutes. Ten microlitres of the standard solution was injected into the HPLC while running each batch of samples.

Proline: Proline was determined using the rapid method developed by Singh *et al*. (1973). One ml of sample, 4 ml of ninhydrin solution (each ml of the ninhydrin solution consisted of 25 mg of ninhydrin, 0.6 ml glacial acetic acid, and 0.4 ml 6 M orthophosphoric acid, and heated to 70 °C until ninhydrin was completely dissolved) and 4 ml of glacial acetic acid were added to 10 ml centrifuge tubes with 1 ml of deionized water. The thoroughly mixed contents of the tube was kept in a 90 °C water bath for 45 min, then cooled to room temperature. The absorbance was measured at 520 nm using a GBC UV/VIS 916 spectrophotometer (GBC Scientific Equipment Pty Ltd., Dandenong, VIC, Australia).

Total soluble amino acids: One ml of 0.1 M sodium acetate acetic acid buffer (pH=4.3) and 1 ml of ninhydrin reagent (5% ninhydrin in ethanol) was added to 1 ml of the sample supernatant. The samples were vortexed, then immersed in a hot water bath (95 °C) for 15 min, and finally cooled to room temperature. Samples were measured at 570 nm using a GBC UV/VIS 916 spectrophotometer.

Estimates on the relative contribution of cytoplasmic solutes to osmotic potential

The relative contribution of the measured solutes to the cytoplasmic osmolality under 320 mM NaCl was made on the following assumptions: (i) cytoplasm comprises 20% of the cell volume (Winter *et al*., 1993; James *et al*., 2006, and references within); (ii) 95% of Na^+ and Cl^- are sequestered in cell vacuoles (Speer and Kaiser, 1991; Di Martino *et al*., 2003); (iii) leaf Cl^- was about 1.2-fold of Na^+ (Fricke *et al*., 1996; James *et al*., 2006); (iv) the osmotic pressure was balanced across the tonoplast, preventing NaCl from leaking back to the cytosol; and (v) most compatible solutes and K^+ were preferentially accumulated in the cytosol rather than the vacuole, under severe saline conditions. The relative contribution of each component was calculated according to its absolute amount in the leaves of salt-tolerant and -sensitive cultivars as elsewhere (Meloni *et al*., 2001; De Lacerda *et al*., 2003; James *et al*., 2006).

Statistical analysis

Data were analysed using SPSS 14.0 for Windows. All results are given as means ±SE. The Student's *t* test was used to calculate the significance of differences between results. Different lowercase letters in each panel of the figures indicate significance at $P < 0.05$ level.

Results

Plant growth and nutritional response to salinity

Similar to our previous reports, 4 weeks of severe salt stress had a strong impact on plant growth, with height, fresh mass and dry mass, all being significantly reduced ($P < 0.05$; Table 1). The effect of salinity, however, differed significantly between barley cultivars, with much better performance of salt-tolerant varieties Numar and ZUG293 after 4 weeks of 320 mM NaCl treatment (Table 1). This difference in growth rate was also reflected in a substantial difference of leaf Na^+ and K^+ content (Fig. 1A, B), where salt-sensitive varieties Gairdner and ZUG403 accumulated significantly higher Na^+ and showed greater K^+ loss compared with salt-tolerant ones ($P < 0.05$). Leaf sap osmolality did not differ significantly between genotypes under control conditions (Fig. 1C), but

Table 1. *Plant height, fresh and dry weight in control and 320 mM NaCl treatment of four barley cultivars differing in salt tolerance* (n=*40 for plant height,* n=*24 for fresh and dry weight*)

Different lowercase letters in each column indicate significance at P <0.05 level.

Cultivar	Plant height (cm)		Fresh mass (g plant^{-1})		Dry mass (g plant^{-1})	
	Control	320 mM NaCl	Control	320 mM NaCl	Control	320 mM NaCl
Numar	55.0±1.3 a	31.5±0.5 a	25.26±1.37 ab	4.40±0.20 a	3.83±0.15 a	0.92±0.04 a
ZUG293	53.4±1.1 a	32.0±0.9 a	23.32±1.45 ab	4.49±0.22 a	3.52±0.22 ab	0.89±0.05 a
Gairdner	54.7±0.7 a	18.1±0.6 c	25.48±1.07 a	2.13±0.24 c	3.06±0.11 b	0.42±0.04 c
ZUG403	56.6±1.3 a	24.0±0.6 b	22.69±1.10 b	2.44±0.18 b	3.57±0.24 ab	0.55±0.04 b

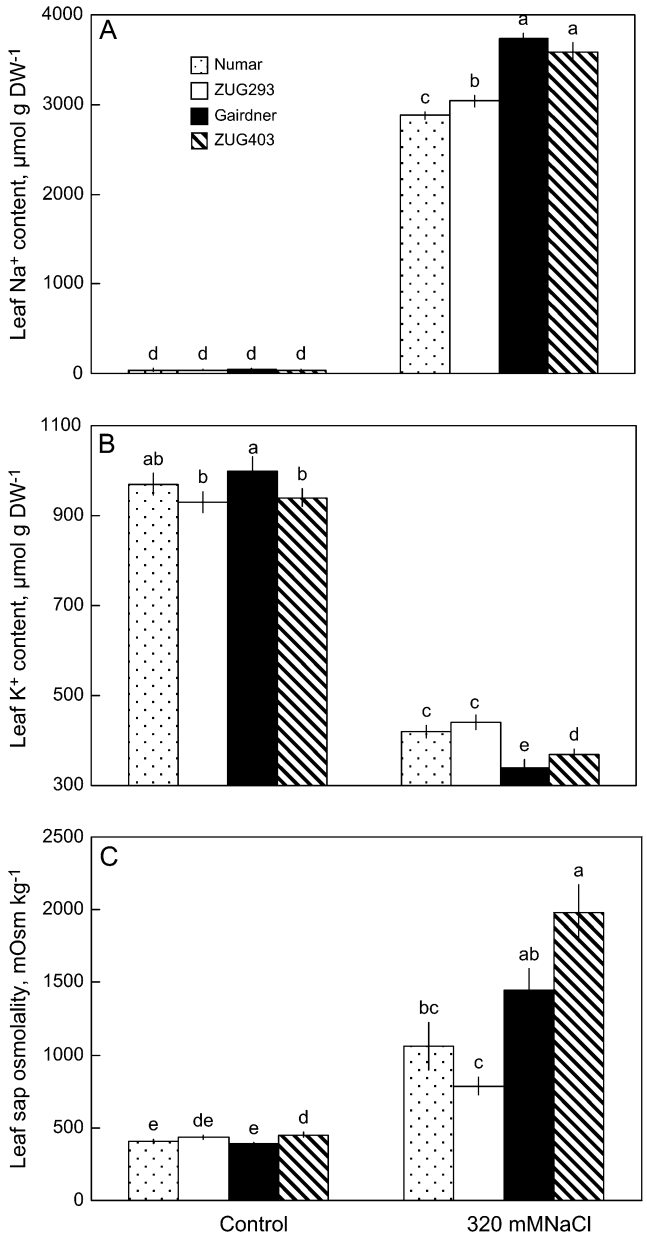

Fig. 1. Comparison of Na^+ (A), K^+ (B) content, and sap osmolality (C) from flag leaves of four barley genotypes in both control and four weeks of 320 mM NaCl treatment. Data are means ±SE (n=4). Different lowercase letters indicate significance at P <0.05 level.

increased under salinity treatment ~ 2 and 4-fold for salt-tolerant and salt-sensitive cultivars, respectively (Fig. 1).

K^+ flux and E_m of salt-tolerant and salt-sensitive genotypes respond differently to ROS

Exogenous application of ROS (H_2O_2) induced a significant K^+ efflux from epidermal cells in the mature region of barley roots (Fig. 2). This H_2O_2-induced K^+ efflux was not instantaneous, as has been found for the acute NaCl treatment (Shabala *et al*., 2003), but rather, it developed gradually reaching peak values after 5–10 min, with the peak K^+ efflux showing some dose-dependency on the amount of H_2O_2 applied (Fig. 2A, B). Potassium flux gradually recovered after reaching its peak, although it always remained as a net efflux. A similar pattern of a slowly increasing ROS-induced K^+ efflux was also observed from *Arabidopsis* roots by Cuin and Shabala (2007*b*) after the application of a $OH^{\bullet}$-generating copper/ascorbate mix. Regardless of H_2O_2 concentration used, salt-sensitive genotypes lost on average ~2.5-fold more K^+ during the first 20 min of oxidative stress (Fig. 2A, B). Consistent with the results of H_2O_2-induced K^+ efflux, E_m of the root epidermis cells was significantly depolarized within the first 15 min of exposure to 10 mM H_2O_2 in all four genotypes (Table 2; Fig. 2B). This H_2O_2-induced membrane depolarization was significantly (P <0.01) smaller (more negative E_m) in salt-tolerant cultivars compared with the salt-sensitive ones (Table 2).

Mitigating effects of glycine betaine and proline on NaCl-induced K^+ efflux

Consistent with our previous reports (Cuin and Shabala, 2005), exogenous application of glycine betaine or proline significantly reduced the extent of NaCl-induced K^+ efflux (Fig. 3A, B), but only in salt-sensitive barley genotypes (31±1.8% and 43±4.6% reduction after 1 h pretreatment for 1 mM and 10 mM of exogenous glycine betaine, and 26±6.2% and 35±8.5% for 1 mM and 10 mM of exogenous proline, respectively; Fig. 3). However, the effect of these treatments on K^+ loss from salt-tolerant cultivars was only marginal (Fig. 3A, B).

Polyols accumulation under saline conditions

Sorbitol, mannitol, and pinitol were detected in both leaf and root tissues using the HPLC technique. The content of each of these components was, on average, several fold higher in roots compared with leaves, regardless of the treatment (Table 3). No clear difference between contrasting varieties was observed. Four weeks of 320 mM NaCl treatment reduced root polyol content in all genotypes except ZUG403. The average reduction for the remaining three cultivars was 30±5.2, 37±6.5, and 44±7.4% for sorbitol, mannitol, and pinitol, respectively. At the same time, sorbitol and pinitol content in the leaves increased by 33±9.2% and 86±18%, respectively, while mannitol levels were essentially unchanged (Table 3).

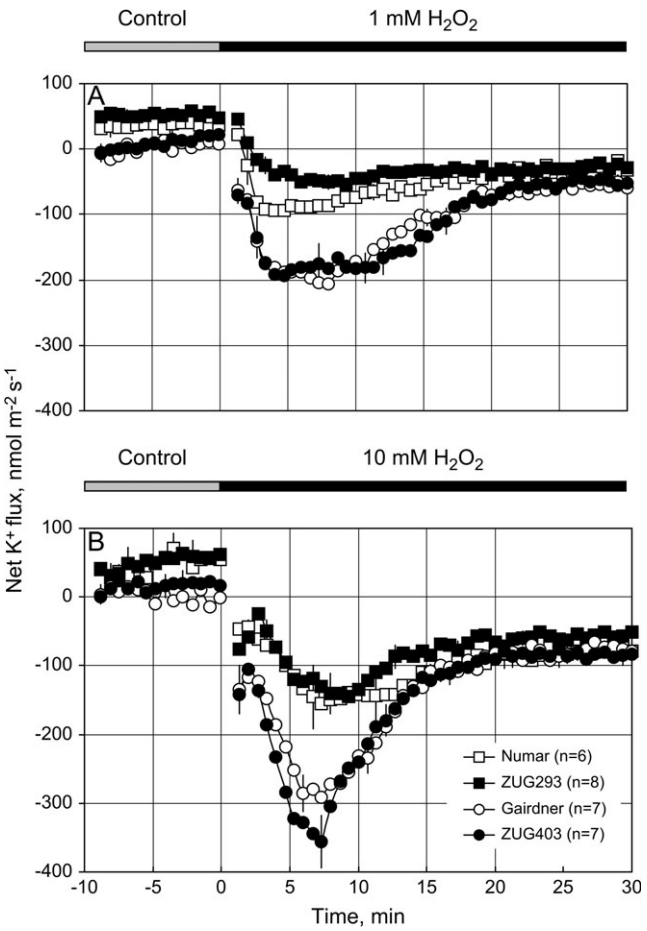

Fig. 2. Transient root K^+ flux responds to a sudden shock of 1 (A) or 10 (B) mM H_2O_2 applied to four barley cultivars contrasting in their salinity tolerance. Data points are averaged at 30 s of K^+ flux recording. Error bars are SE (n=6–8 plants).

Effects of salinity on the total amino acids pool

The total amino acids pool was found to increase in leaves while decreasing in roots after severe salinity treatment (Fig. 4). The two salt-sensitive Gairdner and ZUG403 showed, on average, a 1.8-fold increase in leaf total amino acid content compared with a slight increment for salt-tolerant Numar, while leaf total amino acid content of the most salt-tolerant ZUG293 remained unchanged (Fig. 4A). The effect of salt stress on the total amino acid content in roots was much smaller, with the only significant (P <0.05) decline found for the salt-sensitive cultivar Gairdner (29% reduction; Fig. 4B).

Effects of salinity on glycine betaine and proline accumulation

Four weeks of 320 mM salinity stress significantly increased leaf glycine betaine and proline accumulation in all varieties, but the effect of salinity differed substantially between genotypes (Fig. 5A, B). Salt-sensitive cultivars, on average, accumulated over twice as much leaf glycine betaine and proline than salt-tolerant plants under 320 mM NaCl (P <0.05; Fig. 5A, B). Leaf glycine betaine and proline accumulation correlated negatively (r= –0.89 and –0.94, respectively; P <0.05; Table 5) with the roots ability to retain K^+ under saline conditions (a measure of salt tolerance; Chen *et al*., 2005).

Root glycine betaine was undetectable in both treatments, most likely due to its accumulation primarily in chloroplasts (Robinson and Jones, 1986; Ahmad *et al*., 1987; Nuccio *et al*., 1999). Root proline content in salt-tolerant varieties was twice as high as that of salt-sensitive barley (Fig. 5C). In general, root proline content was substantially lower than in leaves (5-fold and 20-fold difference for salt-tolerant and -sensitive genotypes, respectively; Fig. 5). As such low concentration is unlikely to have any osmoprotective value, the role of proline as ROS scavenger (Xiong *et al*., 2002) is more likely.

Table 2. *Membrane potential of four barley genotypes in control and 5 to 15 min after 10 mM H_2O_2 treatment*

Data are means ±SE (n=10–14). Different lowercase letters in the same column indicate significant difference at P <0.01.

Cultivar	Salt tolerance	Membrane potential (mV)		Depolarization (mV)
		Control	10 mM H_2O_2	
Numar	Tolerant	–130.1±1.92 a	–91.0±2.97 a	39.1
ZUG293	Tolerant	–126.0±2.41 ab	–90.1±3.87 a	35.9
Gairdner	Sensitive	–122.6±1.69 b	–68.8±2.25 b	53.8
ZUG403	Sensitive	–127.9±1.73 ab	–73.5±2.82 b	54.4

Correlation analysis

As one of the early indicators of salt tolerance (Chen *et al*., 2005), NaCl-induced K^+ efflux strongly correlated (P <0.01) with H_2O_2-induced K^+ flux, root proline content, relative fresh and dry mass. Significant correlations (P <0.05) were also found between NaCl-induced K^+ efflux and leaf glycine betaine and proline concentration, relative plant height, and leaf sap osmolality (Table 5). The growth components (fresh and dry mass, plant height) and leaf sap osmolality have also been used as indicators of salt tolerance in our previous work.

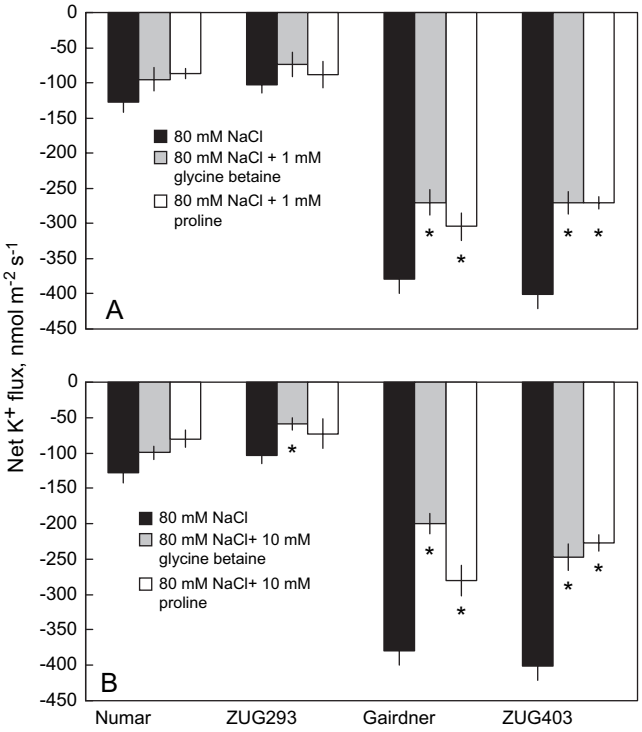

Fig. 3. Effects of 80 mM NaCl and exogenously applied 1 (A) or 10 (B) mM glycine betaine and proline in addition to 80 mM NaCl on root K^+ flux of barley cultivars differing in salt tolerance. All plants were pretreated for 60 min with their respective treatments. Data are averaged over a 15 min K^+ flux recording. Error bars are SE (n=6–10 plants). Statistical significance (P <0.05, t test) of K^+ fluxes within each cultivar is indicated by asterisks.

Discussion

We have previously reported a strong positive correlation between the ability of roots to retain K^+ and salt tolerance in barley (Chen *et al*., 2005, 2007), highlighting the crucial role of intracellular K^+ homeostasis for plant performance under saline conditions. We have also showed that exogenous application of compatible solutes mitigates both NaCl- and ROS-induced K^+ loss (Cuin and Shabala, 2005, 2007*a*, *b*). It has been frequently suggested that ROS-scavenging activity is an important component of salt-tolerance mechanisms (Zhu, 2001). It is also well known that ROS may be efficiently scavenged by osmoprotectants, such as proline and mannitol (Xiong *et al*., 2002; Shabala and Cuin, 2006). This poses the question of whether salt-tolerant genotypes also have a superior ability to withstand oxidative stress and (assuming the affirmative answer) to what extent this trait is related to the accumulation of compatible solutes in plant tissues? These issues are addressed in this study.

Salt-tolerant barley show better tolerance to ROS stress

It is reported in this study that salt-susceptible barley cultivars also had a lower tolerance to ROS (H_2O_2), as shown by the 2–3-fold higher K^+ loss from the root epidermis in the mature region (Fig. 2), and that this difference may be attributed to the various extents of membrane depolarization by ROS stress (Table 2). Intracellular K^+ homeostasis is critical for plant salt

Table 3. *Comparison of leaf and root polyol (sorbitol, mannitol, and pinitol) content of four barley cultivars in both control and 320 mM NaCl treatment*

Data are means ±SE. n=4 for each cultivar and treatment.

	Cultivar	Sorbitol (μmol g^{-1} DW)		Mannitol (μmol g^{-1} DW)		Pinitol (μmol g^{-1} DW)		Total polyols (μmol g^{-1} DW)	
		Control	Salt	Control	Salt	Control	Salt	Control	Salt
Leaf	Numar	18.9±0.5	30.5±1.9	10.2±0.4	7.6±0.6	6.0±0.9	8.3±0.9	35.1	46.4
	ZUG293	27.3±1.1	35.5±1.4	9.8±1.8	9.5±1.1	7.3±0.5	12.9±1.1	44.4	57.9
	Gairdner	30.4±3.1	32.5±3.1	9.7±1.2	12.5±1.1	7.1±0.8	17.2±4.3	47.2	62.2
	ZUG403	30.8±2.5	30.6±4.8	15.4±0.7	13.3±1.8	8.0±0.7	n.d.[a]	54.1	43.9
	Mean	26.8	32.3	11.3	10.7	7.1	12.8	45.2	52.6
Root	Numar	80.2±4.5	48.7±9.7	37.9±3.1	22.8±1.1	28.6±3.6	15.0±0.5	146.7	86.5
	ZUG293	57.4±5.4	40.4±3.4	30.2±2.3	16.1±2.5	29.2±1.9	20.7±2.1	116.7	77.2
	Gairdner	67.9±7.4	53.5±5.3	28.9±0.4	21.8±4.3	35.2±3.1	16.3±2.2	132.0	91.6
	ZUG403	38.0±2.1	85.1±6.0	21.9±2.8	33.4±4.0	20.3±2.5	18.5±1.9	80.2	137.1
	Mean	60.9	56.9	29.7	23.5	28.3	17.6	118.9	98.1

[a] n.d., Not detected.

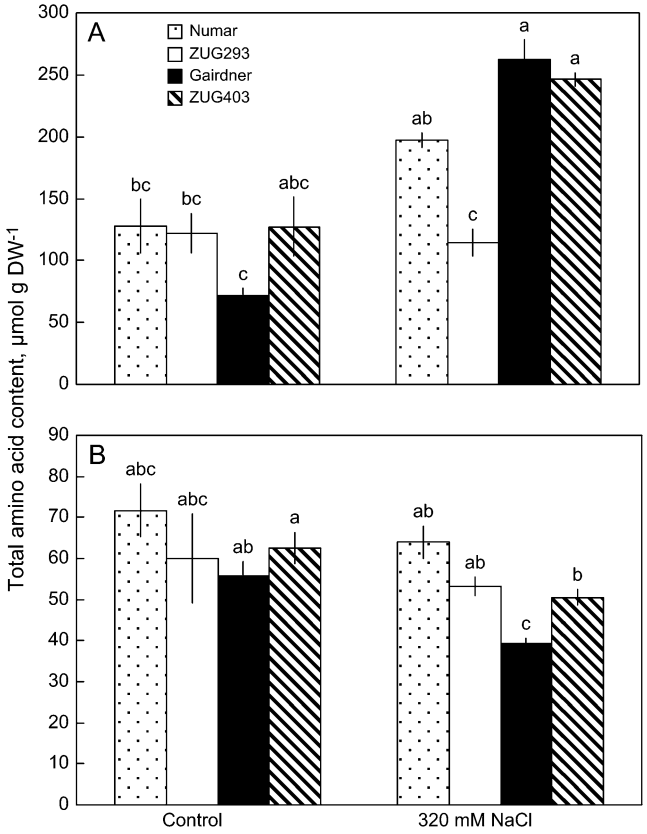

Fig. 4. Effects of 320 mM NaCl treatment on leaf (A) and root (B) total amino acid content among four barley genotypes differing in salt tolerance. Data are mean ±SE (n=4). Different lowercase letters indicate significance at P <0.05 level.

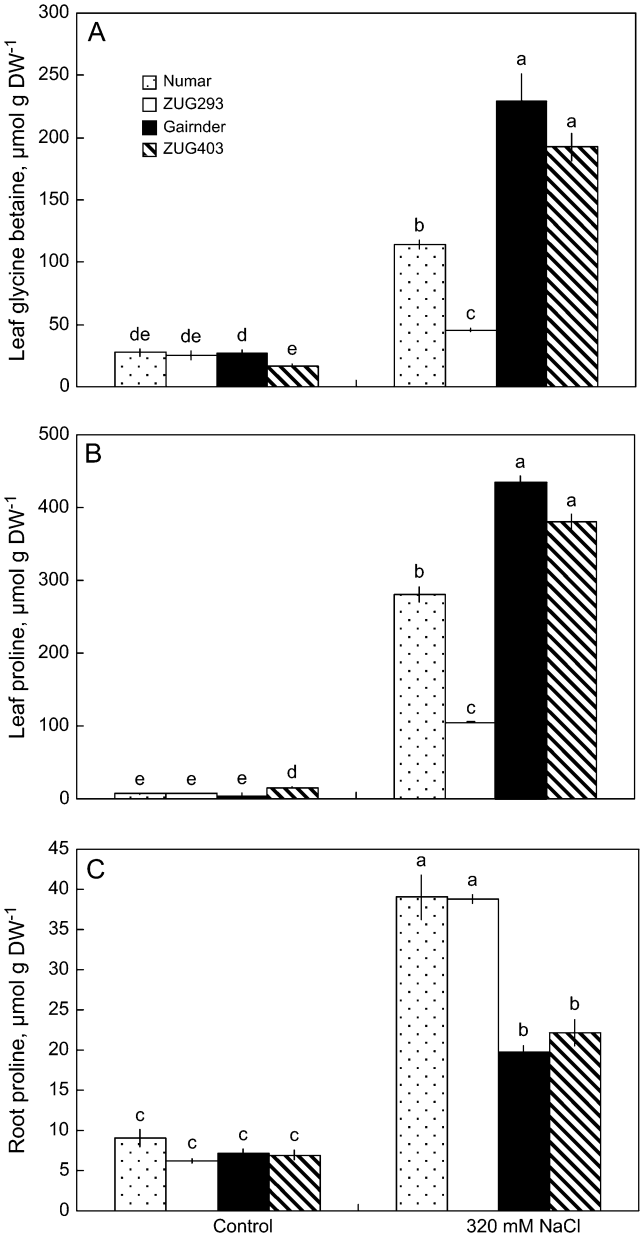

Fig. 5. Effects of 320 mM NaCl treatment on leaf glycine betaine (A), leaf proline (B), and root proline (C) content in four barley genotypes contrasting in salinity tolerance. Glycine betaine and proline content in control condition is also shown in each panel. Data are mean ±SE (n=4). Different lowercase letters indicate significance at P <0.05 level.

tolerance (Zhu *et al*., 1998; Maathuis and Amtmann, 1999; Carden *et al*., 2003; Peng *et al*., 2004; Chen *et al*., 2005; Shabala *et al*., 2006) and may be achieved by different means. ROS-activated K^+ channels have previously been described in many animal systems (Kourie, 1998) and ROS-stimulated K^+ efflux has been observed in root cells of various plants (Demidchik *et al*., 2003, 2007; Shabala *et al*., 2006; Cuin and Shabala, 2007*b*). Under saline conditions, the balance between ROS production and scavenging is broken, causing a rapid increase in ROS level (Apostol *et al*., 1989; Mittler, 2002; Apel and Hirt, 2004) and concomitant K^+ efflux (Shabala, 2006; Cuin and Shabala, 2007*b*; Fig. 2). Also, NaCl-induced plasma membrane depolarization will cause activation of depolarization-activated Ca^{2+} channels (DACC), leading to an increase in cytosolic free Ca^{2+} and a consequent stimulation of NADPH oxidase and elevated ROS generation (Demidchik and Maathuis, 2007). The superior ability of salt-tolerant cultivars in preventing ROS-induced K^+ loss from their roots is suggestive of an intrinsically better defence system in these genotypes. For instance, NaCl-induced oxidative stress caused an increased H_2O_2 accumulation due to inefficiencies in H_2O_2 scavenging in salt-sensitive potato cultivars, so they produced larger amounts of the antioxidant proline to compensate for the H_2O_2 scavenging (Fidalgo *et al*., 2004). This could also partially explain the higher leaf proline levels in salt-sensitive barley. It will be interesting to extend this study to a wider range of genotypes so as to investigate the extent to which this trait reflects the ability of salt-tolerant barley to prevent ROS-induced K^+ loss by maintaining better enzymatic and non-enzymatic defence systems.

Relative contribution of solutes to cytoplasmic osmolality under severe salt stress

The dramatic increase in leaf sap osmolality (Fig. 1C) in plants subjected to salt stress was largely the result of high accumulation of Na^+ (Fig. 1) and Cl^- in the leaf cells and salt-induced water loss (Chen *et al*., 2005). However, in the cytoplasm, the relative contribution of K^+ to the osmolality was the highest amongst all the solutes studied (Table 4). In salt-tolerant varieties, it constituted about half of cytoplasmic osmolality. In salt-sensitive genotypes, however, this figure was substantially lower (Table 4), leading to the requirement for salt-sensitive plants to synthesize at least twice as much cytoplasmic glycine betaine and proline as salt-tolerant ones. The contribution of amino acids (excluding proline) and polyols to osmotic potential were minor in both salt-tolerant and -sensitive genotypes (Table 4).

NaCl-induced K^+ efflux in salt-susceptible cultivars is more sensitive to exogenously applied glycine betaine and proline

Exogenously supplied glycine betaine and proline significantly reduced the magnitude of NaCl-induced K^+ efflux in the two salt-sensitive genotypes (Fig. 3). However, this mitigating effect was not significant in the salt-tolerant varieties (Fig. 3). This difference could be due to a differing regulation by exogenous glycine betaine and proline of the various ion channels mediating NaCl-induced K^+ efflux between salt-tolerant and salt-sensitive genotypes. Increased ROS scavenging is the most obvious candidate. However, both proline and glycine betaine were equally effective in ameliorating ROS-induced K^+ leak from sensitive genotypes (Fig. 3). At the same time, among the three major types of compatible solutes measured in this study (proline, glycine betaine, and polyols), polyols are reportedly the most effective ROS scavengers, followed by proline, while glycine betaine is thought incapable of scavenging free radicals (Smirnoff and Cumbes, 1989; Orthen *et al*., 1994; Matysik *et al*., 2002; Shabala and Cuin, 2006). Thus, some other mechanisms such as membrane integrity protection and increasing structural stability of ion transporters may also contribute to this differential regulation. In practical terms, it is prudent to use this high response of salt-susceptible barley to explore the possibility of supplying exogenous glycine betaine and proline by either foliar sprays or by seeds priming as a means of ameliorating NaCl stress.

Table 4. *Relative composition of inorganic and organic solutes in the leaf cytoplasm of salt-tolerant and -sensitive genotypes exposed in 320 mM NaCl for 4 weeks*

Data are averaged from two cultivars in each column (see text for more details).

Solutes	Salt-tolerant lines (%)	Salt-sensitive lines (%)
Glycine betaine	6.2	13.5
Proline	13.9	24.4
Amino acids (except for proline)	7.2	6.0
Polyols	3.6	3.0
K^+ and its charge balancing anions	49.7	33.1
Na^+, Cl^-, and unknown solutes	19.5	20.0

Table 5. *Linear correlation between NaCl-induced K^+ flux (80 mM NaCl) and other parameters determined in this study*

Parameter	NaCl-induced K^+ flux[a]
Leaf sap osmolality	0.91*
Relative plant height	0.94*
Relative fresh mass	0.98**
Relative dry mass	0.99**
H_2O_2-induced K^+ flux	0.98**
Leaf glycine betaine content	–0.94*
Leaf proline content	–0.89*
Root proline content	0.99**

[a] Significant at * P <0.05, ** P <0.01.

Roles of polyols and amino acids in barley salt tolerance

In root tissue, soluble sugars (sucrose, glucose, and fructose) or glycine betaine were below the detection limit (data not shown) of the HPLC. Also, proline accumulation was over 10 times lower than that in leaves. Polyols and amino acids appear to be the major compatible solutes within root tissues (Table 3; Fig. 3, 4). Polyols are mainly synthesized in mature leaves (source tissue) as primary products of photosynthesis and transported to roots (sink tissue) (Noiraud *et al*., 2001). This is reflected by a root polyol content more than twice that of leaves, regardless of salt treatment (Table 3). Polyols may also act as ROS scavengers, thus protecting enzyme activities and membrane integrity (Smirnoff and Cumbes, 1989; McCue and Hanson, 1990; Bohnert *et al*., 1995; Shen *et al*., 1997; Noiraud *et al*., 2001).

The much higher total amino acid content increase in leaves of salt-sensitive varieties (Fig. 4A) may be also indicative of these plants' greater need for ROS scavenging. A higher Na^+ accumulation and a more pronounced K^+ loss in leaves of salt-sensitive genotypes (Fig. 1A, B) results in reduced photosynthetic efficiency (Chen *et al*., 2005), so generating greater oxidative stress in light-exposed leaves. Thus, more amino acids (especially proline) may be needed to mitigate the ROS stress in salt-sensitive cultivars.

Hyperaccumulation of glycine betaine and proline under high salinity does not improve salt tolerance in barley

The importance of K^+ homeostasis in barley salinity tolerance has been investigated in our previous studies

(Chen *et al*., 2005, 2007; Cuin and Shabala, 2005, 2007*a*). The present data are consistent with these reports. Salt-tolerant varieties had a much higher K^+ contribution towards cell osmotic adjustment under saline conditions (50% versus 33% for salt-sensitive varieties). As a result, salt-sensitive cultivars needed to synthesize high levels of glycine betaine and proline to compensate for this difference so as to balance the intracellular osmotic potential (Table 4). These findings are consistent with reports of higher leaf proline in salt-sensitive genotypes of other species (Colmer *et al*., 1995; Balibrea *et al*., 1997; Lutts *et al*., 1999). It therefore raises the question as to whether the large amount of glycine betaine and proline are actually beneficial for salt adaptation (Rabe, 1990; Lutts *et al*., 1999). Compatible solutes are non-toxic for cytosolic accumulation in plants, but are energetically more expensive. Surviving in saline condition imposes the cost of both excluding salt and its compartmentation within the cell. However, this cost is relatively small compared with that needed to synthesize organic solutes (Yeo, 1983; Raven, 1985). It can be calculated that salt-sensitive Gairdner consumed about 4.5-fold of ATP and nitrogen source for synthesizing glycine betaine and proline than salt-tolerant ZUG293. This could be a partial cause of the reduction in growth (Table 1) and higher leaf sap osmolality (Fig. 1C) of salt-sensitive genotypes. Gross measurement of compatible solutes, however, has its disadvantages due to difficulties in its detection within different cell compartments. For instance, glycine betaine is accumulated in chloroplasts to protect leaves from salt stress. Much higher leaf glycine betaine accumulation might also indicate the inefficiency of glycine betaine sequestration (Leigh *et al*., 1981) in chloroplasts of salt-sensitive genotypes. Specific aspects of such intracellular compartmentation are outside the scope of the current study and should be addressed in a separate investigation.

Conclusion

This study shows that superior K^+ retention and efficient usage of compatible solutes are crucial components for barley salt tolerance. Salt-tolerant cultivars maintained both smaller NaCl- and a ROS-induced K^+ efflux. Micromolar amounts of compatible solutes are sufficient for salt-tolerant cultivars to survive in severe salinity. By contrast, hyperaccumulation of compatible solutes in salt-sensitive barley did not ameliorate the sensitivity to salt, but, instead, appeared to be a symptom of injury.

Acknowledgements

This work was supported by GRDC (UT8) and DEST grant to M Zhou, ARC Discovery (DP0449856) and DEST grants to S Shabala. We would like to thank Professor Guoping Zhang for generously providing barley seeds.

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